EX3

Recombinant DNA

A Short Course

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Rearranging Germ-Line DNA Segments to Form Antibody Genes

For several decades an enormous number (perhaps in the millions) of different antibody (immunoglobulin) molecules have been known to exist, each characterized by a unique site that can bind to specific molecular determinants (antigens). Many immunologists thought initially that all antibodies were made of the same polypeptide chains and that their uniqueness arose from the way their newly synthesized identical polypeptide chains folded around the respective antigens. This theory was proved wrong. Each antibody has its own amino acid sequence, and each antibody-producing cell (plasma cell) makes only one antibody. At first, this was a disturbing discovery because it seemed to imply that a separate gene would have to exist for each separate antibody. If so, perhaps a large fraction, if not the majority, of the vertebrate DNAs would have to be devoted to coding antibody molecules. But such speculations could not be tested until protein chemists established the basic structure of the antibody molecule.

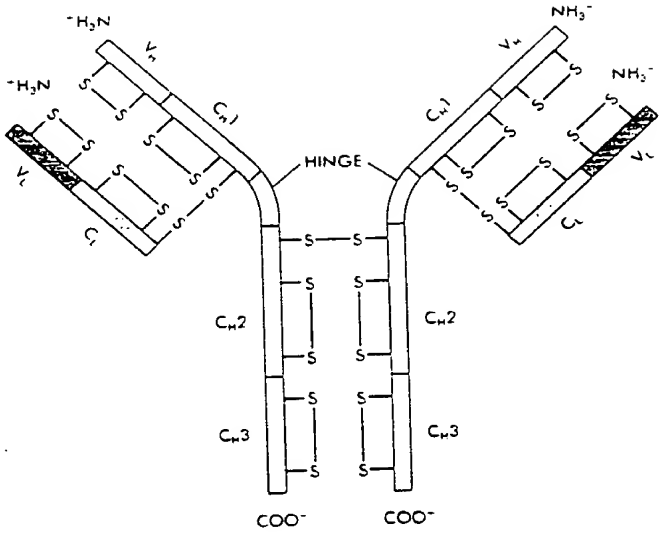

Figure 9-1
The structure of an antibody protein. Two light chains (color) and two heavy chains (white) are held together by disulfide bonds. The light chains and the heavy chains each contain one variable unit (V_L or V_H) at their amino-terminal ends. The light chains also contain one constant unit (C_L); the heavy-chain constant portion has four domains (C_H1, C_H2, C_H3, and the hinge region).

The Basic Structure of Antibody Molecules Is Established

The first insights began to emerge in the early 1960s, when it was realized that the fundamental antibody unit consists of two identical light (L) chains of molecular weight 17,000 and two identical heavy (H) chains of molecular weight 35,000, held together by disulfide bonds (Figure 9-1). (The terms "light" and "heavy" refer to the differences in the molecular weight of the chains.) Each such four-chain unit contains two identical binding sites for antigens, with a site being formed partly by specific amino acids of the light chain and partly by specific heavy-chain amino acids. Once the basic antibody layout had been established, the amino acid sequences of the component light and heavy chains were determined by using the homogeneous antibodies made by specific myeloma cells. Myelomas are cancerous antibody-producing

(plasma) cells, and in any one animal all the cells of a myeloma tumor are the descendants of one original cancer cell. This explains why all the antibody molecules from any one myeloma have the same amino acid sequence.

Both light- and heavy-chain sequences vary from one type of antibody to another, but in a way that no one would have predicted initially. Although each chain has unique sequences, almost all of this specificity is restricted to about 100 amino acids at the amino-terminal ends (the variable, or V, regions). Half of each light chain and three-quarters of each heavy chain have almost identical sequences (the constant, or C, regions) (Figure 9-1, page 117).

Separate Genes for V and C Segments Are Proposed

To account for the constant and variable portions of the chains, William Dreyer and Claude Bennett at the California Institute of Technology put forward a bold hypothesis in 1965. They proposed that the V and C regions are coded by separate genes, and that in the germ line the C-region segments (C_L for the light chain, C_H for the heavy chain) are each coded for by only one gene, but that the V regions (V_H and V_L) are coded for by many thousands of genes. Dreyer and Bennett further proposed that a functional antibody is formed when genetic recombination in the precursor to the plasma cell brings one of the V genes next to its respective C gene to yield V_LC_L and V_HC_H genes. This perceptive hypothesis won few early converts because it flew in the face of the general belief that the arrangement of DNA within a given chromosome was effectively immutable, except at meiosis during the formation of the sex cells.

Messenger RNA Probes Are Used to Obtain Support for the Joining of V and C Genes

It was impossible to test this V–C joining hypothesis until there were direct molecular probes that might identify the putative V and C genes. In the first experiments, which were done between 1974 and 1976, mRNA probes that were more than 50 percent pure were made from myeloma cells. These mRNA probes were mixed, under conditions favoring hybridization, with unfractionated homologous myeloma DNA to see if it was possible to count the number of genes for the single antibody type that was present in a given myeloma cell. The answer for at least one light-chain mRNA probe was that there were a very low number of genes, and perhaps only one.

Such experiments, however, could not distinguish V from C sequences, nor could they indicate differences in the relative locations of V and C sequences in embryonic cells compared with myeloma cells. To do that required a way to cut up the total myeloma and embryonic cell DNAs into reproducible pieces, a procedure that became possible only with the ready availability of restriction enzymes. Using them, Susumu Tonegawa at the Basel Institute of Immunology in Switzerland observed in the spring of 1976 that V and C sequences that were linked together on the same DNA restriction fragment from an antibody-producing mouse myeloma cell were not similarly linked together in embryonic DNA (Figure 9-2). This classic experiment was done with necessarily impure mRNA probes because of the still-effective prohibitions against cloning cDNA molecules. But as soon as the first suitable cDNA vector was approved, the appropriate cDNA probes for V and C regions were made and their specificity directly determined by DNA sequencing. These regions were then used for selecting genomic segments that carried the respective antibody genes. Possession of such reagents has revolutionized our understanding of the molecular basis of antibody diversity.

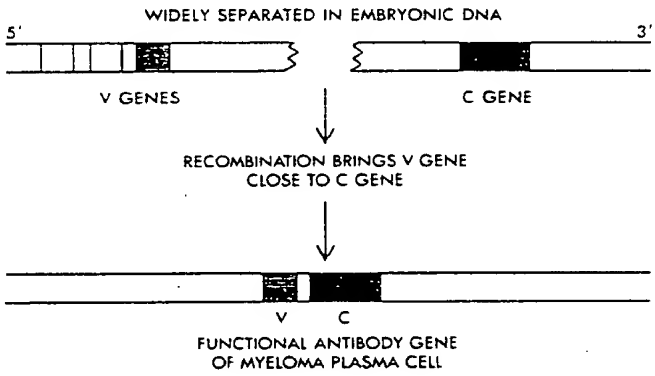

Figure 9-2
Functional antibody genes are produced by genetic recombination. The V and C genes in embryonic myeloma DNA were discovered to be widely separated, whereas they were found close together in mature myeloma antibody-producing cells.

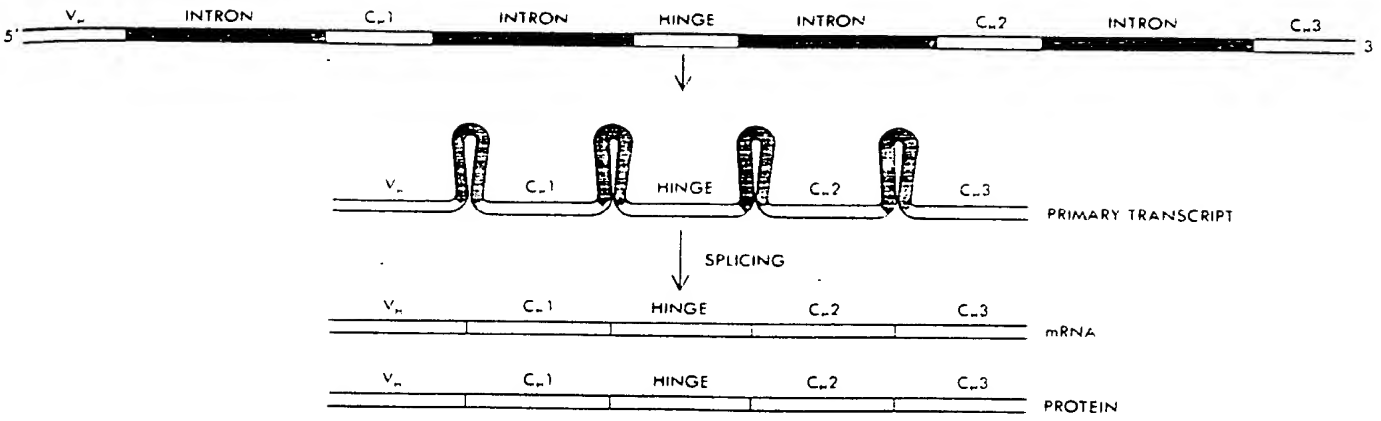

Figure 9-3
The protein domains of immunoglobulin heavy chains are separated by introns.

Functional Antibody Genes Are Isolated from Myeloma Cells

The cDNA probes that established the nature and number of antibody genes were made from specific myelomas whose antibody products had already been sequenced. Direct comparisons were thus possible between the nucleotide sequences of functional antibody genes and the amino acid sequences of the antibodies they specified. Many introns were found immediately, and, most importantly, most were located at junctions between functional domains. In the light chain, an intron separates almost all of the amino-terminal leader sequences from the V segment, and a second intron divides the V from the C sequences. Within heavy-chain genes, introns separate functionally related domains (in other words, they separate exons coding for domains) more extensively. Each of the three domains of the C_H protein is clearly delineated by introns, as is the so-called hinge region lying between the first and second C_H domains (Figure 9-3). All of these observations have supported the hypothesis that proteins have evolved by the rearrangement of exons.

Embryonic Cells Are Sources of Unjoined V and C Genes

The structures of the V and C segments before they are joined to create functional antibody genes were revealed by cloning the appropriate genomic DNA segments from embryonic cells and hybridizing them with probes specific for the V and C regions. C-Region probes invariably were very specific, whereas V-region probes often hybridized to many different V genes. Such cross-hybridization reflects the fact that the V regions of different antibodies often differ by only a few amino acid substitutions. Now we have evidence for at least 200 V_L genes and an equal number of V_H genes. Note that because the specificity of an antibody is determined by both its V_L and V_H components, the number of potentially different antibodies is at least $V_L \times V_H$, or $200 \times 200 = 40{,}000$.

In contrast to the multiplicity of V_L and V_H genes, there exist only two C_L genes (C_λ and C_κ), located on different chromosomes, and a single cluster of linked C_H genes. In the mouse, this C_H cluster is located on chromosome 12 and consists of eight genes that are spaced out over some 200 kilobases (kb) of DNA. The separate C_H genes reflect different functional roles for their gene products, with, for example the C_μ gene coding for the early-appearing immunoglobulin M, and the several C_γ genes coding for immunoglobulins that have generally higher specificity and that predominate in the later stages of an immune response.

Multiple J (Joining) Segments Are Attached to Genomic C (Constant) Segments

The number of potential light chains is greatly increased by the presence of a cluster of related, but not identical, J (for joining) segments that reside upstream of each C_L gene. The number of

potential heavy chains is likewise increased by a J-segment cluster located upstream from each C_H cluster. The linkage of a V segment to a C segment may occur next to any of these J segments, and, depending on which J segment is used, a different group of amino acids will be found inserted between the amino acids encoded by the V segment and those encoded by the C segment. RNA splicing thus occurs in such a way that only the J segment used in the V–C joining event is retained (Figure 9-4). How splicing can be so regulated remains totally mysterious, as does the nature of the events that join the V and C genes together.

The joining event itself is slightly variable, which creates additional diversity at the V–JC combining site. The site occurs, probably not by chance, within the nucleotides coding for amino acids that help form the cavity into which antigens bind. One model for DNA joining postulates that the respective V and C segments, initially located far apart on the same DNA molecule, are brought together by a recombination process that eliminates the intervening sequences. The sequences at the ends of the V and JC segments are complementary and could allow formation of hydrogen-bonded hairpin loops that would align the appropriate bases ready for cutting and rejoining. There is some experimental support for this proposal.

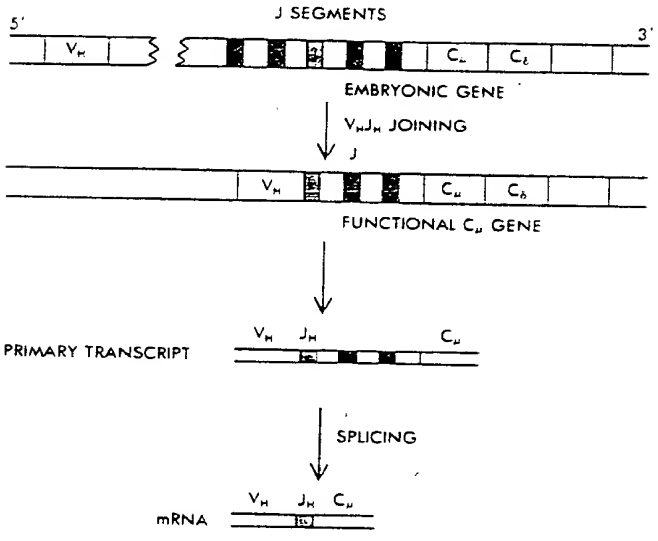

Figure 9-4
A V_H gene is linked to a C_H gene by means of a J (joining) segment that is located in a cluster of such segments upstream of the C_H genes. After the initial recombination event, RNA splicing removes all of the other J segments to produce the mature mRNA.

Three Discontinuous Regions of DNA Code for Heavy-Chain Amino Acids

At first it was believed that heavy-chain formation followed the same pattern of events as light-chain formation. But when the appropriate germ-line V_H and J_HC_H regions were cloned, sequenced, and compared to the sequences found in the respective myeloma heavy chains, it became apparent that a second group of internal amino acids were not coded by either the V_H or J_HC_H segments but had to arise from a third DNA segment. This third segment, given the name D (for diversity), is sited between the V_H and J_HC_H segments. It consists of a tandem multigene family of related sequences. The existence of multiple D segments, any one of which has the possibility of being inserted into the functional heavy-chain gene, still further increases the potential number of heavy-chain specificities ($V_H \times D_H \times J_H$) (Figure 9-5). Like the J-encoded amino acids, the D-encoded amino acids help to form the antigen-binding sites, so the diversity they create is likely to be biologically useful.

A DNA Elimination Event Allows a V_H Gene to Be Attached to Two Different C_H Genes

Recombinant DNA procedures have also swiftly solved the puzzle of how a given heavy-chain variable gene (V_H) can be attached first to a constant segment (C_μ) that is characteristic of the immunoglobulin M class, and then, during the later immune response, can transfer its linkage to a constant segment (C_γ) characteristic of the immunoglobulin G class. No change of immunological specificity occurs during this transfer, because the heavy-chain types differ only in their "constant" components, all of which are coded by a group of genes clustered together on the same chromosome. How this switch happens became crystal-clear as soon as the appropriate C_μ and C_γ genes were cloned and their sequences were compared with those of a functional gene coding for a known γ-class heavy chain (MOPC 141).

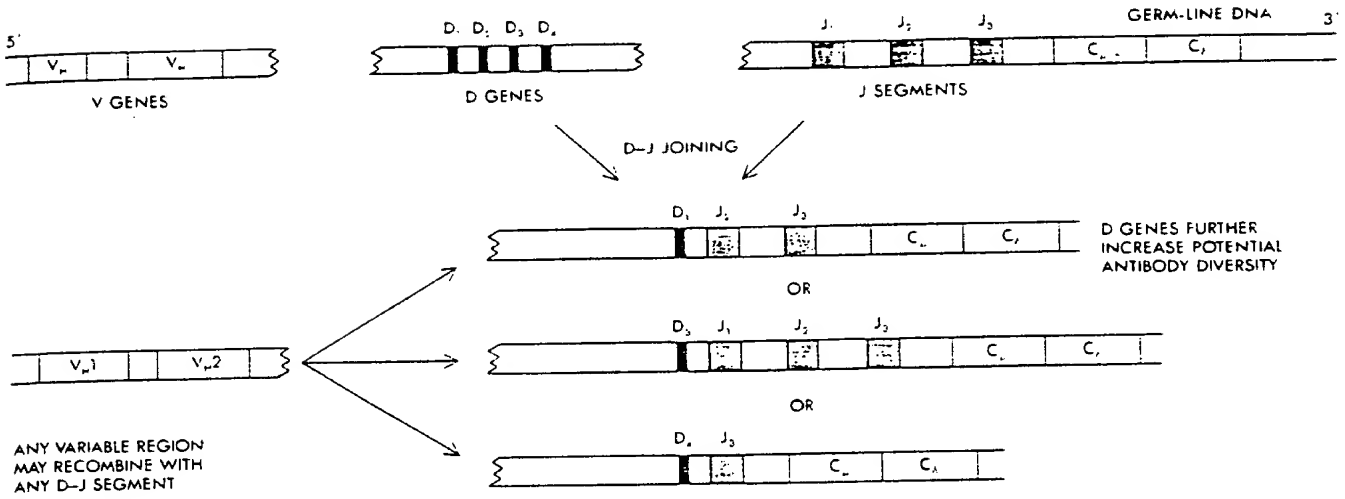

Figure 9-5
D (diversity) segments join to J segments to create additional potential diversity in antibody molecules.

The key observation was the finding of J-segment bases in the gene coding for the heavy chain of MOPC 141, despite the absence of any J segment near the corresponding embryonic C_γ gene. In contrast, several J segments were found at the beginning of the embryonic C_μ gene, one of which exactly corresponded to that observed in the functional C_γ gene of MOPC 141. Two recombination events are therefore necessary to generate a functional gene for γ-class heavy chains. The first joining event attaches a V_H gene to the C_μ gene at one of its flanking J segments, thereby allowing synthesis of a μ-class heavy chain. This synthesis continues until a second recombination event removes most of the C_μ gene sequences and links the previously joined V_HJ_H segment to the intron sequences flanking a C_γ gene. This leads to the synthesis of a γ-class heavy chain whose J-coded sequences bear witness to the prior $V_HJ_HC_H$ arrangement (Figure 9-6).

Alternative Splicing Allows Single Cells to Make Both μ- and δ-Class Heavy Chains with Identical V_H Segments

Recombinant DNA procedures have also clarified another previously puzzling observation—namely, that one cell can simultaneously make heavy chains that are of two different types but that contain the same variable region. The initial explanation proposed for this situation was that one type of heavy chain might be translated from a very stable long-lived mRNA that persisted in the cytoplasm long after the corresponding gene had been eliminated. Analysis of the structure of the genes involved with recombinant DNA techniques, however, suggested an alternative. The variable region (V), a joining region (J), and the two constant regions C_μ and C_δ were found to be contiguous. The whole complex might thus be translated into a

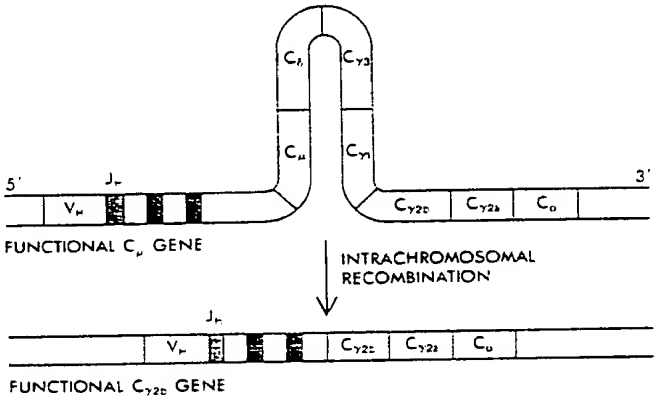

Figure 9-6
Genetic recombination changes a functional μ-class gene to a functional γ-class gene. This recombination event occurs after the formation of the initial V_H–J_H link.

large precursor RNA molecule from which, by differential splicing, either one or the other of the constant regions is eliminated (Figure 9-7). This mechanism was confirmed with cDNA probes prepared from the appropriate mRNA. Differential splicing of a common precursor RNA generates two distinct heavy-chain mRNAs. A dual-splicing potential also determines whether certain classes of antibodies are bound to the plasma membranes of the cells in which they are made or are secreted to the outside.

Somatic Mutations Provide a Further Source of Immunoglobulin Diversity

Before the existence of recombinant DNA, there was seemingly endless debate as to whether the specificity for antibody genes lay largely in germ-line DNA segments or whether most diversity was created by somatic mutations that occurred during the multiplication of antibody-producing cells and their precursors. With the discovery of V and (D)JC joining it became unambiguously clear that much antibody diversity was carried in germ-line DNA segments. Virtually simultaneously, however, it was found that the exact amino acid sequence of many antibodies does not precisely correspond with that predicted by their respective germ-line sequences. The first direct evidence for somatic diversification came from analysis of the mouse light-chain variable regions. Of 19 λ proteins examined, 12 had the germ-line sequence while 7 others had one to three amino acid differences. Since then, somatic diversification has also been found in V_H segments, and significant proportions of antibody variability must now be ascribed to somatic mutational events. Although these mutations occur both in the DNA sequences that control the specificity of antigen binding (i.e., V regions) and in those that code for the so-called "framework" (C) regions, most mutations are observed to affect antigen binding. Equally important "mutant" antibodies are largely restricted to the later stages of the immunological response, in

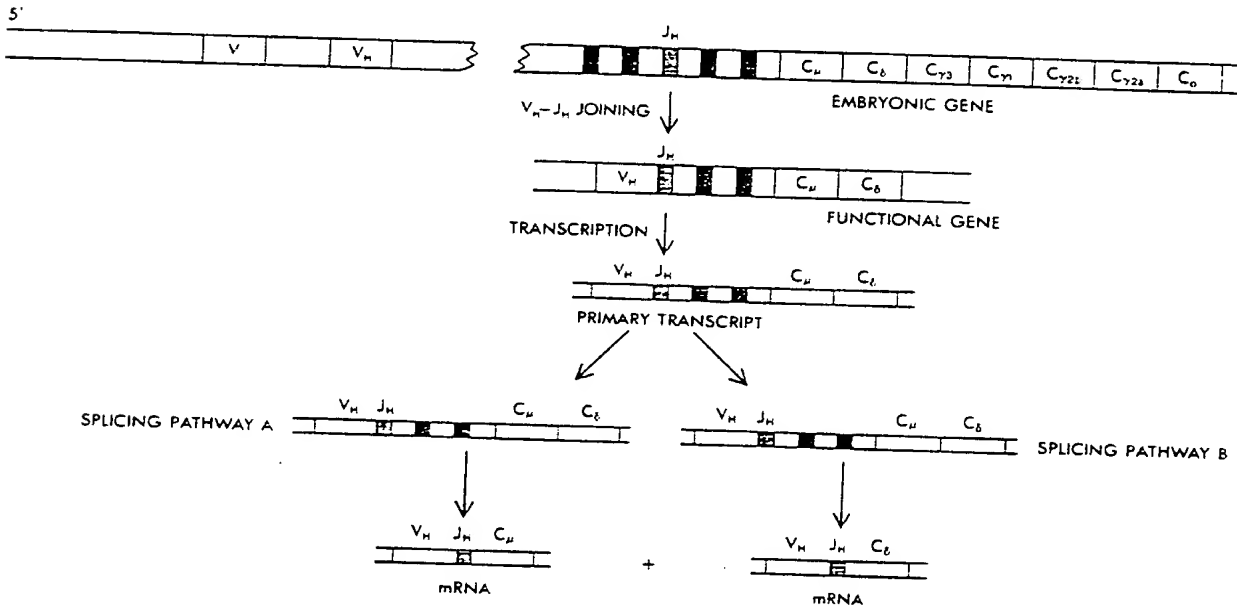

Figure 9-7
Alternative splicing can generate antibody molecules with different heavy-chain determinants. Here a large precursor RNA molecule is differentially spliced so as to produce two different kinds of RNA: that coding for a μ-class heavy chain and that coding for a δ-class heavy chain.

which C_γ- (or C_α-) chain synthesis predominates. It has thus been asked whether the antibodies produced by somatic mutations are more efficient (whether they bind antigen better) than the early-appearing antibodies whose specificity comes entirely from germ-line sequences. Though immunological theory predicts that this should occur, preliminary experiments have failed to support this idea.

Establishing the Genes of the Major Histocompatibility Complex (MHC) Proteins and Their Protein Antibodies Through Gene Cloning

Some 30 years ago, the rejection of foreign skin grafts (transplantations) in mice was found to depend on a group of proteins (H2) that recognized the skin cells as "nonself." It was determined that these proteins were coded by closely linked genes that were mapped to a region of chromosome 17. With time, the number of these so-called "histocompatibility proteins" was found to be far greater than was first perceived, and the "major histocompatibility complex" (MHC) is now known to code for three very different classes of proteins. Class I consists of the genes for the highly polymorphic H2 transplantation antigens (in humans, the HLA antigens), which are expressed on virtually all cell surfaces, as well as the genes for other surface molecules restricted to specific types of differentiated cells (for example, blood-forming cells). The class II (immune-response) genes encode a specific group of cell surface molecules that are present only on lymphocytes and that control the extent of specific immunological responses. Several components of the complement system that links an immune response to the desired destruction of an unwanted foreign cell by lysis constitute the class III genes.

Our understanding of the exact chemistry of these MHC proteins has lagged far behind our understanding of the chemistry of the immunoglobulins themselves, and only with the advent of cloning procedures is a comprehensive picture of the MHC structures beginning to emerge. In general, the class I H2 and H2-like proteins contain a 45,000-dalton transmembrane protein attached

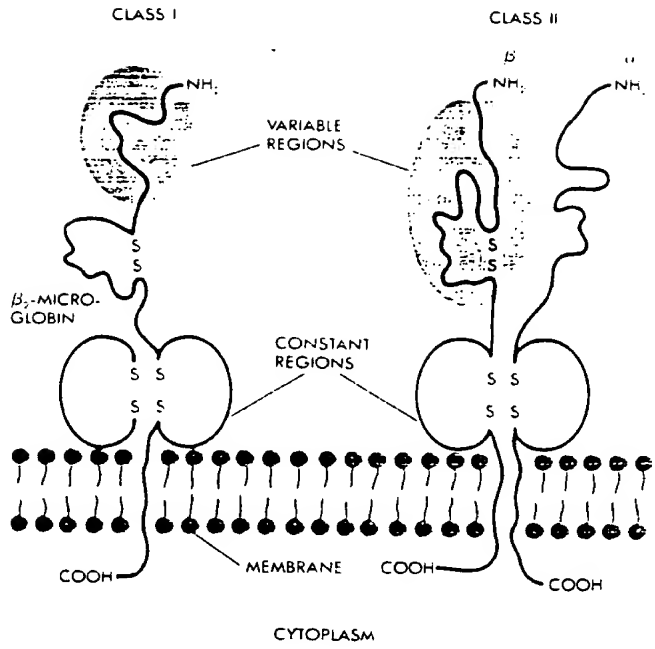

Figure 9-8
Major histocompatibility complex proteins and their protein antibodies. Class I and class II MHC proteins have variable and constant regions in their cytoplasmic domains. The constant regions show some homology with the constant regions of immunoglobulin molecules. Class I MHC proteins are associated with another protein called β_2-microglobulin.

noncovalently to a 12,000-dalton protein, β_2-microglobulin, that is separately encoded on chromosome 2. Each 45,000-dalton chain contains three external domains comprising approximately 90 amino acids, a transmembrane region of approximately 40 amino acids, and a short cytoplasmic region of about 30 amino acids (Figure 9-8). This structure reflects the exon–intron arrangement, with separate exons encoding the signal leader peptide, each of the exterior domains, the transmembrane region, and the three regions of the cytoplasmic component.

The organization of the class I genes along chromosome 17 has been revealed through cloning 40-kb fragments of mouse DNA into cosmids and looking for overlaps between clones contain-

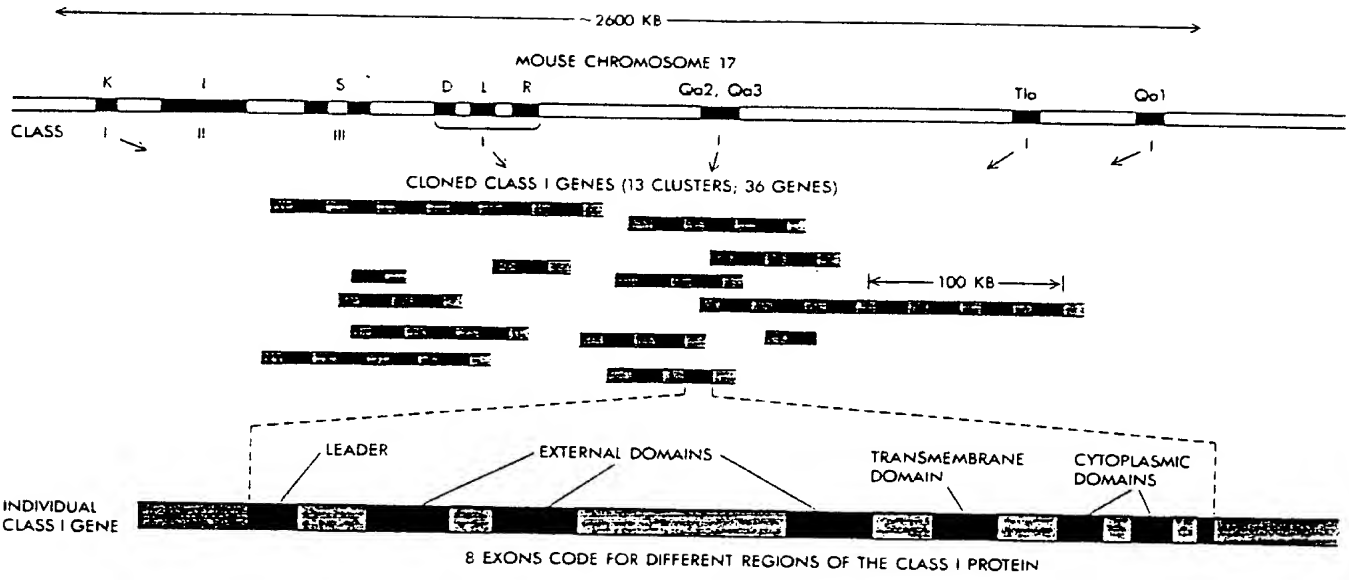

Figure 9-9
The genes coding for MHC class I, II, and III proteins are linked on mouse chromosome 17. The 36 class I genes have been cloned on cosmids and were found to occur in 13 clusters containing varying numbers of genes.

ing class I genes (Figure 9-9). So far, 36 class I genes encompassing some 837 kb of DNA have been found. Now the class II and class III genes are in the process of being mapped, and through chromosome walking the complete molecular map of the mouse MHC complex could be obtained over the next several years.

Recombinant DNA analysis has thus already profoundly affected immunological research. The separate origins of immunological diversity are in the process of being sorted out. Equally important, by being able to go directly to the MHC genes, which control the extended nature of immunological responses, we are much closer to explaining the phenomenological descriptions that have constituted cell immunology than we would have been if we had been limited to the biological approaches of the cellular immunologist.

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